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APPLICATION NUMBER: *60/510,344*
FILING DATE: *October 10, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/32933*

Certified by

Jon W Dudas

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U.S.PTO 19249 60/510344 101003

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ER 272749709 US

INVENTOR(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
Ge Ming	Lui	55 South Kukui Street Apt 2810 Honolulu, HI 96813

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Corneal Cell Culturing and Transplantation

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☑ Customer Number: 38551

OR

☐ Firm or Individual Name					
Address					
Address					
City		State		Zip	
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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 18 ☐ CD(s), Number _____

☐ Drawing(s) *Number of Sheets* _____ ☐ Other (specify) _____

☐ Application Date Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees.

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☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____

TYPED or PRINTED NAME Ge Ming Lui, PhD

TELEPHONE 808 949 2208

Date October 10, 2003

REGISTRATION NO. _____ *(if appropriate)*

Docket Number: _____

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This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: **Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	
Filing Date	October 10, 2003
First Named Inventor	Ge Ming Lui
Examiner Name	
Art Unit	
Attorney Docket No.	

METHOD OF PAYMENT *(check all that apply)*

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		X		=
Independent Claims		- 3** =		X		=
Multiple Dependent						=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

SUBMITTED BY

Name (Print/Type)	Ge Ming Lui	Registration No. (Attorney/Agent)	38551	Telephone	808 949 2208
Signature				Date	Oct 10, 2003

(Complete *(if applicable)*)

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Patent 1: Corneal Cell Culturing and Transplantation

BACKGROUND ON VARIOUS STEPS

Title: 1A1 - Method of Obtaining and Culturing Cells for Corneal Cell Transplantation

Background

Brief description of what this patent describes:

This patent describes the improved methods of dissecting, seeding and subsequent propagation of pure culture of human corneal endothelial cells on extracellular matrix.

What problem(s) does this address?:

This method of culturing human corneal endothelial cells (HCEC) will solve the problems:

1. HCEC cells can only be seeded at high density (2000-5000 cells/square mm) therefore limiting the possibility to start a primary culture from small specimen.
2. HCEC cells cannot be passaged continuously at low seeding density (50-100 cells/square mm) and therefore limiting the ability to expand the stock for storage and future use.

How is this different from what's been done by others?

This method of culturing HCEC on extracellular matrix enable the establishment of primary cultures from small specimens (100-500 cells) and also to expand these primary cell colonies via serial passage into large quantity of cells for transplantation and cryostorage for future use.

What future applications might this have?:

This method of cell culture can be used for the establishing and serial culturing of other cell types of human origins such as neurons, pancreatic beta cells, and chrondrocites.

Specific Methods and/or Compositions

Step-by-step preferred method and/or description of composition:

1. Establishing primary culture.

Dissection of corneal endothelial cells: Corneal buttons or rims were placed endothelial side up, rinsed with PBS several times. A shallow cut was made inside the trebacular meshwork to expose the underlining Descement's membrane. By using a jeweler's forcep, the layer of endothelial cells were pulled off the stroma and placed facing downward onto an ECM coated 35 mm tissue culture plate. 0.5 ml of media containing 15% fetal calf serum, antibiotics and 50 ng of bFGF was added to the plate without allowing the tissue to detach from the ECM. The plate was incubated in 37 degree C and 10% CO2 incubator for 24 hours, and then another 1 ml of media was added. The plate was left in the incubator without disturbance for 4-5 days. The media was changed on day 5 and 50 ng/ml of bFGF was added every other day until outgrowth of 100 to 200 cells was observed.

Preparation of coated ECM plates for primary corneal endothelial culture and subsequent passaging. Bovine corneal endothelial cells (BCEC) in culture will be seeded onto dishes in a DME-H16 medium containing 10% FCS, 5% CS, 5% Dextran, 300 ug/ml glutamine, 2.5 ug/ml Amphotericin B, and 50 ng/ml bFGF. At confluency,(7-10 days post seeding), the dishes will be treated with 20 mM NH4OH at a volume sufficient to cover at lease 2/3 of the plate. After 5 minutes of shaking in a mechanical shaker, the NH4OH will be aspirated and the dished rinsed 5 times with PBS. The dishes will be stored at 4 degree C at lease

a week prior to use in order to eliminate any surviving BCEC. Laminin and fibronectin will be dissolved in distilled water at 100 ug/ml. Type IV collagen will be dissolved in 0.6% v/v acetic acid/water. Laminin, fibronectin, and type IV collagen will be added to the ECM plates as needed for culture purpose.

2. Expanding cell line.

Serial culture of human corneal endothelial cells(HCEC). When the initial explant reached a population of 200-300 cells, they were removed from the dish with a solution containing 0.05% trypsin, 0.02% EDTA in normal saline (STV solution). To facilitate the process, the cells in STV was incubated at 37 degree C for 2-3 minutes, until >90% of the cells were round up. The STV was then removed, and the cells further incubated at 37 degree C for 1 minute. By using a 1 ml Pipetman, the cells were dislodged from the dish by repeated washing with medium containing 15% FCS. The primary HCEC thus harvested were added onto a 60 mm tissue culture dish coated with BCE-ECM. At no time during this procedure the cells were undergone centrifugation. 5 ml of culture media containing 15% FCS was added with 50 ng of bFGF. The media was changed every other days and bFGF was added at the time of medium change until the culture was confluent.

3. Expansion of stock HCEC cells.

The confluent first passage cells were split into 10-20 6mm dishes coated with BCE-ECM. Under the same culture conditions as previously mentioned, the cells were grown to confluency. The cells were then trypsinized by using STV solution at 37 degree C for 5 minutes. The STV was then aspirated and the cells allowed to round up at room temperature (25 degree C). 5 ml of media containing 15% FCS was added to neutralize the STV. The cells were knocked off the dish by using a 1 ml Pipetman and the cell suspension was collected.

4. Freezing of HCEC stock.

For each of the 5 ml of HCEC collected, 0.5 ml of DMSO was added to the cell suspension. Each 1.1 ml of the mixture was aliquoted into a 1.5 ml cryopreservation tube to yield an approximate 1 million cells per vial final concentration. The vials were then put into a Styrofoam box and let stand in a -80 degree C freezer for 24 hours. After 1 day, the ampoules were transfer into liquid nitrogen for long term storage.

Alternative steps or materials to address potential problems or if certain materials are not available:

If the extracellular matrix is not available, an artificial matrix can be generated although such material will not be as effective in promoting the growth and morphological integrity (hexagonal shape) of the HCEC.

To generate the artificial matrix, fibronectin, laminin and RGDS are dissolve at 100 ug/ml in distill water, collagen type IV is dissolved at 1 mg/ml in 0.01% acetic acid. Basic fibroblast growth factor is dissolced at 100ug/ml in bovine serum albumin (0.05% w/v). All the materials are mixed together in a 15 ml centrifuge tube and swirled gently to avoid bubbling. The mixture is then incubated at 4 degree C for two hours.

To coat the tissue culture dishes, the mixture is diluted 1:10 with phosphate buffered saline, and then 1 ml of the solution is added to a 35 mm dish and store at 4 degree C for 1 hour. Prior to use the solution is aspirated and the cell suspension will be added to the dish.

Better methods or compositions if new materials or complementary methods become available:

At present the extracellular matrix coated plates can only be stored in PBS at 4 degree C. This sometimes lead to bacterial or fungal contamination due to spilling of the storage

medium. A method of drying the coated dishes without losing biological activity is a big improvement.

Inventive Contribution, Improvements

(List all the points of this idea you feel are novel, critical, and/or patentable.)

1. The combination of extracellular matrix coated dishes and addition of basic fibroblast growth factors to the culture medium for establishing primary culture of HCEC from small tissue specimen.
2. The combination of extracellual matrix coated dishes and addition of basic fibroblast factor, epidermal growth factor to the culture medium for expansion of HCEC from low density (100-500 cells/square mm) to confluent culture in serial passaging.
3. The use of artificial generated matrix materials coated dishes (fibronectin, laminin, RGDS, collagen type IV, basic fibroblast growth factor, bovine serum albumin) in lieu of extracellular matrix coated dishes for the propagation of HCEC.
4. Use of dissection method to pull the corneal endothelium away from the stroma along the Descemet's membrane when establishing primary HCEC culture instead of using enzymatic method to dissociate the cells from the specimen.

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Title: 1A2 -Method of Dealing with HLA-typing In Corneal Cell Transplantation

Background

Brief description of what this patent describes:

This patent describes ways to minimize the expression of histocompatibility antigens in cultured HCEC through gene masking or cell banking.

What problem(s) does this address?:

Despite the eye is known as a "immune privileged site", there is still a 25% graft rejection rate in native corneal transplantation. To generate cultured HCEC with depressed expression of allograft reactivity will decrease the rate of graft failure due to immune rejection.

How is this different from what's been done by others?:

The current approach is to use fetal or neonatal cells for corneal endothelial replacement, but it is still reported to associate with 25 to 50% primary failure rate.

What future applications might this have?:

The gene masking or knock out concept can be used to create other universal donor cell lines in other organ.

Specific Methods and/or Compositions

Step-by-step preferred method and/or description of composition:

1. Use of neonatal cells.

Despite the eye being immune-privileged, there is still 25% rejection in corneal transplantation. It is known that cells derived from 4 month or younger donors do not express Class I HLA antigens. In addition, corneal button from such young donors are unsuitable for transplantation the curvature of the cornea is

too large. An advantage of using cell lines from neonatal donors is that the cell line established from such primary cultures has a longer in vitro life span than cells derived from older donors. Since it has been reported that transplanting neonatal corneal cells in animal model still associated with 25 to 50% failure rate, more studies must be done to determine if the failures were due to immune rejection or other physical or physiological perimeters.

2. Gene knockout.
The idea is to mask or delete the sequence in the genes of the HCEC culture which code for the Class I HLA antigen via transfection or other gene manipulation. This approach carries the risk that other functional entities may be altered or deleted, thus creating a transformed cell line.

3. HLA Matching.
This approach requires a step in cell banking. The ability to create HCEC primary cultures using the BCE-ECM method allows the establishment of a HCEC bank by cryostoring cells from many donors. All the banked cells can be typed and their HLA types recorded. When the need for corneal endothelial replacement arise, the HLA type for the recipient will also be typed and then matched against those of the stored HCEC in the cell bank. The cell line with the closest HLA type will be used for the cell transplantation.

Alternative steps or materials to address potential problems or if certain materials are not available:
Stem cell can be used in lieu of neonatal cells to test the hypothesis that cells which do not express cell surface immune epitopes will not elicit graft rejection in corneal endothelial cell replacement.

Better methods or compositions if new materials or complementary methods become available:
If a large number of HCEC cell lines can be established from different donors and their HLA typing information is documented, then trying to match the HLA type of the recipient with one of the cell line in the cell bank may be a simpler approach to avoid allograft reactivity.

Inventive Contribution, Improvements
(List all the points of this idea you feel are novel, critical, and/or patentable.)
1. Generation of universal donor HCEC lines by gene masking or gene knockout.
2. To establish a HCEC bank to match HLA type with recipients of corneal graft to reduce the rate of graft failure due to immune rejection.

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Title: 1B - Method of Transporting and Packaging Cells

Background
Brief description of what this patent describes:
This patent describes a method to create a carrier system for transportation of HCEC in tissue culture to a different location for immediate use in corneal endothelial cell replacement surgery.

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What problem(s) does this address?:

If HCEC is shipped by frozen ampoule in dry ice, the cells are not ready to be transplanted for at least 3 to 4 days after arrival. To circumvent this problem cells can be grown in tissue culture flask and shipped to the destination. This way the cells still need to be trypsinized and reseeded onto the corneal button. Another way of transporting the cells is to grow them on biodegradable polymer sheet. They can be directly applied onto the denuded cornea immediately after arrival.

How is this different from what's been done by others?:

HCEC are mostly transported in frozen ampoule. When shipped as stock in culture, they are grown in tissue culture flasks and transported as such.

What future applications might this have?:

This biodegradable polymer can be modified for carrying other human cell types for transplantation in other parts of the eye and also other organs.

Specific Methods and/or Compositions

Step-by-step preferred method and/or description of composition:

Cell Transporting & Packaging

1. Ampoule.

The frozen HCEC stock are stored in liquid nitrogen. Each ampoule contain approximately 1 million cells immersed in 1 ml of culture medium (15% FCS and 10% DMSO). When defrosting these cells, they are added directly to a 6mm culture dish with 5 ml of media without centrifugation. The dish of cell will be incubated at 37 degree C for 30 minutes, then the media will be aspirated and replaced with fresh media. The cells will be maintained in the incubator for at least 24-48 hours before they can be trypsinized and used for seeding denuded corneal buttons. The ampoule can be shipped in Styrofoam box containing dry ice. After arriving at the destination, it has to be stored at -80 degree C (for no more than 7 days) or in liquid nitrogen for long term storage.

2. Sheet of coated cells, transfer onto biodegradable material.

Instead of shipping the HCEC in frozen ampoules, cells can be shipped in a mono-layer grown in detachable membranes. In this case a biodegradable material which can be coated with cell adhesion proteins or BCE-ECM will be preferred. The HCEC will be grown to confluency in the membrane, which will than be deposited into a flask filled with the culture medium. It can then be packaged and shipped overnight to the user. After arrival, the cell layer can be directly applied onto the denuded cornea via suturing or glue, thus making it ready for transplantation. If the cells are not used for surgery right away, it can be deposited into a tissue culture dish and start another culture.

3.Precoated cornea.

If available, the corneal button from human donor can be denuded and replaced with cultured HCEC (details for denudation and coating will be discussed in later section). The coated cornea will be maintained in incubator for at lease 24 hours prior to shipping to prevent cell detachment due to lower temperature during transportation (usually 25 degree C). The coated cornea can be package in corneal storage medium similar to those used in storing donated cornea. The coated cornea can be used for surgery within a period of 3-4 days.

Alternative steps or materials to address potential problems or if certain materials are not available:
The biodegradable polymer has to be biocompatible and not excessively swelling when it is submerged in fluid. An additional advantage is for it to be strong enough to withstand suturing. Otherwise gluing onto the cornea can be the alternative. If such polymer is not readily available, other biological membrane such as ammiontic membrane can be tested.
Better methods or compositions if new materials or complementary methods become available:
An ideal polymer for this purpose, besides being biodegradable, will be one in a semi-solid state which can be coated with extracellular matrix or Diamond-Like-Carbon (DLC). This modification enables the coated polymer to act as carriers for cells derived from neural origin for cell transplantation and other applications.

Inventive Contribution, Improvements
(List all the points of this idea you feel are novel, critical, and/or patentable.)

1. A sheet of biodegradable polymer with HCEC cells grown on it can be used as carrier for shipping the cells.
2. HCEC grown on a sheet of biodegradable polymer can be applied directly onto denuded cornea button by suturing or gluing in corneal endothelial replacement surgery.
3. Modification on the surface of the polymer by coating with ectracellular matrix, DLC, adhesive proteins, or growth factors can make this carrier useful for other cell types, especially nerve cells.

****

Title: 1C - Method of Creating a Pre-Coated Cornea for Corneal Cell Transplantation

Background
Brief description of what this patent describes:
This patent describes a method to protect a denuded cornea coated with cultured HCEC for the purpose of storage, transporting, and under surgical manipulation.
What problems does this address?:
Cultured HCEC seeded onto denuded cornea sometimes detach from the surface during surgical procedure (suturing), or during transportation.
How is this different from what's been done by others?:
This problem has not been previously addressed.
What future applications might this have?:
The addition of cytoprotective agent (Healon) into the cornea storage medium may create a new generation of this product.

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Specific Methods and/or Compositions

Step-by-step preferred method and/or description of composition:

Precoated Cornea

1. Protection storage of cornea (Healon).

In comparison to native cornea button, coated (or resurfaced) cornea endure a short storage period (3-4 days instead of up to 7 days in the case of native cornea). A good technique to lengthen the storage period of a coated cornea is to use cyto-protective agents such as Healon to prevent cell detachment after coating. When the Healon is conjugated with bFGF, it can maintain the coated cells in the storage medium for 7-10 days, which is similar to storing native cornea button.

In this approach, 0.5 ml of Healon (conjugated with 100 ng of bFGF) will be added to the cell suspension during the coating process. After the incubation , the cornea will be rinsed with fresh media and deposited into a cornea storage medium with 2 ml of Healon (conjugated with 500 ng of bFGF). The corneal button can then be shipped.

2. Cryostorage (may not be practical).

In order to store cornea button in liquid nitrogen, an anti-icing agent such as DMSO or ethylene glycol must be added to the freezing media to prevent the rupturing of cells in the button. Unfortunately, all of the known anti-icing agent are toxic to some extent so that the absorption into the stroma during defrosting may be a potential health hazard. Until a new non-toxic anti-icing agent can be identified, this approach is not very promising.

Alternative steps or materials to address potential problems or if certain materials are not available:

Healon is a patented product. If it is not available as an ingredient for a new product, other cytoprotective agent such as polycarbophyll, carboxymethylsephadex, hyuaronic acid can be used.

Better methods or compositions if new materials or complementary methods become available:

When basic fibroblast growth factor is conjugated with Healon, it works better to preserve the integrity of the cultured HCEC coated onto the denuded cornea.

Inventive Contribution, Improvements

(List all the points of this idea you feel are novel, critical, and/or patentable.)

1. Use of cytoprotective agent(s) to overlay the cultured HCEC seeded onto denuded cornea to minimize cell detachment during surgical manipulation.
2. Addition of cytoprotective agent and growth factors into the cornea storage medium for shipping and storage of cornea coated with HCEC.

****

Inventor: Ge Ming Lui
Patent Owner: Cellular Bioengineering, Inc.
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Title: 1D - Method of Denuding and Coating an Existing Cornea for Corneal Cell Transplantation or Correction

Background

Brief description of what this patent describes:

This patent describes methods to remove the native corneal endothelial cells from the corneal button and the application of adhesive proteins/growth factors mixture to coat the denuded cornea button prior to seeding cultured HCEC on top.

What problem(s) does this address?:

The problems addressed by this patent are:

1. How to completely remove the native corneal endothelial cells without damaging the underlying membrane.
2. How to ensure a firm attachment of the cultured HCEC onto the denuded cornea>
3. How to achieve a firm attachment of the HCEC in the shortest possible incubation time.

How is this different from what's been done by others?:

The commonly used method for removing the existing corneal endothelial cells is by means of swiping the endothelial layer with a wetted microsponge. The approach may lead to uneven removal of cells and damage in the underlying membrane in certain areas. We use a chemical agent in addition to the mechanical swiping to remove the endothelial cells uniformly and completely.

At the same time, no attempt has been made to pre-coat the denuded corneal button prior to seeding the culture HCEC onto it, therefore making it difficult for the cells to form firm attachment in a short time.

What future applications might this have?:

This same application (adhesive proteins/growth factors mixture) can be used to coat the artificial cornea made of biopolymers prior to seeding cultured HCEC cells onto its surface for transplantation.

Specific Methods and/or Compositions

Step-by-step preferred method and/or description of composition:

Denuding & Coating of Existing Cornea

Denuding.

a. Triton X - Due to the gentle nature of this detergent, a range of concentrations can be used for the denudation of the native endothelial cells. The concentration ranges from 0.01% to 1 % v/v of Triton X in PBS can be used for this purpose. The cornea button will be placed endothelial side up in a polymethylmetharylate holder. Triton x will be added in sufficient volume (about 700 ul) to completely cover the cornea without spilling. After 5 minutes of incubation at 25 degree C, the Triton X will be aspirated and the cornea washed 10 times with PBS.

b. Ammonium Hydroxide - Ammonium hydroxide proves to be very effective in removing the native corneal endothelial cells in a short time (2-5 minutes) and at a low concentration (20 mM). However, due to its strong alkali property sometimes the underlining Descemet's membrane as well as the stroma show signs of damage. This method was preferred in animal studies since a lot of the animals used showed regenerative endothelial cells from the host, which never occurs in human. A method to reconstitute the

underlining Descemet's membrane by using adheresive proteins (see later section) may make this method of denudation an alternative to the Triton X method.

c. Hypoosmotic hydrolytic - In this approach the cornea will be placed endothelial side up in a holder and 700 ul of distill water is added to the corneal button. After 15 minutes of incubation at 25 degree C, 300 ul of water will be aspirated. A cellulose micro sponge will be used to gently sweep the entire wetted endothelium. The corneal button will then be washed 3 times with PBS.

d. Mechanical - This method provide the most rapid approach in removing the native endothelium but suffers from two disadvantages. The first being it is hard to remove all the native endothelial cells evenly. This is important since the need for corneal endothelial replacement arise only because the old endothelial layer was damaged or diseased and therefore required total removal. The second weakness of this method is that if the sweeping was done too harshly, it may also damage the Descemet's membrane. In this case reconstitution with attachment proteins is warranted.

Coating.

a. Recreate Descemet's membrane by using attachment factor.
For this purpose 4 attachment proteins, 2 growth factors and 1 conjugating polyglycan will be used. 3 of the attachment factors, laminin, and RGDS will be dissolved in PBS at a concentration of 500 ug/ml. Collagen type IV will be dissolved in 0.1 M acetic acid at a concentration of 500 ug/ml. To create conjugation of bFGF and EGF to the polymer, in this case we can use either heparan sulfate or polycarbophyll, the bFGF is dissolved at a concentration of 10 ug/ml and EGF dissolved at concentration of 100 ug/ml in PBS. Polycarbophyll is also dissolved in PBS at a concentration of 1 mg/ml. Then equal volume of bFGF, EGF and polycarbophyll (1 ml of each) will be mixed together and swirled gently for 2-3 minutes. The mixture is allowed to stand in room temperature for 30 minutes. In the mean time 1 ml each of laminin, fibronectin and RGDS will be mixed with 3 ml of collagen type IV, also agitated gently to avoid excess foaming of the protein. The growth factors mixture will then be added to the adhesion proteins mixture and the resulting solution be mixed by hand gently for 3 minutes. This solution of reconstituted ECM will be kept at 4 degree C for 2 hours minimum to allow further interaction prior to be used for coating of denuded cornea. To coat the denuded cornea button, the cornea is placed endothelial side up in a holder and the denudation step as previously described will first be performed. After rinsing with PBS extensively, 700 ul of the adhersive-growth factors mixture will be added to the corneal button. The cornea will then be incubated at 4 degree C for 20 minutes and the mixture be aspirated. Depending on the degree of damage to the Descmet's Membrane caused by the denudation step, the coating procedure can be repeated 2-3 times to enhence the deposition of the ECM-like material and thus obtaining better results of HCEC adhesion and function.

b. Use growth factor: EGF & FGF (conjugated).
This part was covered in the previous section.

c. Healon(hyualonic acid). Protects cell and cornea stroma. Healon will be used for two purposes:

1. To prevent the cornea button from excessive swelling during the cell coating process. It is observed that during the seeding of HCEC onto the denuded cornea, the cornea usually becomes swollen into 2 to 3 times its original thickness due to absorption of the culture media during the incubation period. However, if the denuded cornea was first treated with adhesion proteins as previously described, then 300 ul of Healon was added prior to the seeding of HCEC cells onto the denuded cornea for the duration of the incubation period, then the cornea swelling will be reduced by 80-90%.

2. To prevent the newly attached HCEC from dislodging as a result of surgical manipulation. Since the incubation period for HCEC coating onto the denuded cornea is as short as 30 minutes, some of the cells may not be firmly attached when the cornea is subsequently trephined and sutured. These surgical manipulations may cause detachment of loosely adhered cells into the anterior chamber causing fibrin formation as well as insufficient cell coating of the cornea. To overcome this problem, after the cell coating

step is completed and the cornea is rinsed , 700 ul of Healon is added to the cornea button and then the cornea can be used for transplantation. The Healon served as a cytoprotectant throughout the surgery.

3. Surgery

a. Always use 1/2mm larger corneal button.
This is feasible if cornea from another donor is used for the surgery. However, if the recipient's own stroma is used for the cornea endothelial replacement surgery, then the same size cornea button will be used.

b. Always use heparin to wash interior chamber (prevent fibrin formation).
It is observed during the opening of the anterior chamber (by way of trephining out the damaged cornea button), bleeding into the anterior chamber sometimes occurred. This will cause formation of fibrin clot post surgically (in animal at lease) and sometimes leads to graft rejection. A good practice is always to rinse the anterior chamber with heparin injection after opening the cornea prior to suturing back the replacement cornea regardless of whether bleeding occurred or not.

c. Fill anterior chamber with elastic viscous agent (Healon).
After rinsing with heparin, the anterior chamber will be filled with Healon for the duration of the surgery. The viscosity of the Healon will help to support the cornea in position during the suturing process. It also serve as protectant for the newly coated HCEC.

d. Suturing: 8 uninterrupted at 180 degree opposite to fix. Then continuous to prevent the rupturing of the cornea, both interrupted and running suture techniques will be employed at the same time.

e. Close suture knot inside of eye.
To prevent irritation of the eye post surgically.

Alternative steps or materials to address potential problems or if certain materials are not available:

If donor cornea buttons are not sufficient to fulfill the demand for corneal endothelial cell replacement, artificial corneal buttons (made from biopolymers which are biocompatible and strong enough to undergo suturing) can be used as substitute for corneal stroma onto which the HCEC cells be seeded and transplant. Coating with a cocktail of attachment proteins/growth factors in this case becomes more essential to ensure a satisfactory attachment of the cultured cells.

Better methods or compositions if new materials or complementary methods become available:

If a new biopolymer which is high in tensile strength and will not swell excessively when immerge in liquid media can be synthesized, and the polymer is tested biocompatible to be used as artificial cornea, then the adhesive proteins/growth factors mixture can be incorporated into the polymer during synthesis. This will generate a ready to use artificial corneal button.

Inventive Contribution, Improvements

(List all the points of this idea you feel are novel, critical, and/or patentable.)

1. Improved method for denuding the native cornea without damaging the underlying membrane.

2. Composition of adhesive proteins/growth factors cocktail as pre-coating material to facilitate HCEC attachment to denuded cornea.
3. Use of the adhesive proteins/growth factors mixture to coat the artificial cornea to enhance HCEC attachment.
4. Incorporation of the adhesive proteins/growth factors mixture into a new synthetic biopolymer to make a ready to use artificial cornea button.

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October 6, 2003

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Re: Draft Claims for Application 1
Corneal Cell Culturing and Transplantation
Our Ref. No.: 1003-0001

This draft patent application describes the improved methods of dissecting, seeding and subsequent propagation of pure culture of human corneal endothelial cells on an extracellular matrix.

1. The method of non-enzymatic harvesting and *in vitro* culturing corneal endothelial cells for transplantation comprising dissecting corneal endothelial cells from a tissue source; growing said corneal endothelial cells at a low density in a range of about 100 to 500 cells per square millimeter in a primary culture system comprising extracellular matrix coated (ECM) culture plates for a period of time; passaging said cells into a secondary culture system wherein the secondary culture system is comprised of an ECM coated culture plates and the addition of sufficient cellular growth factors until the cells are grown to confluency; and wherein said cells have grown to confluence and are then harvested from the second culture system in sufficient quantities to be useful in transplantation to a subject *in vivo*.

2. The method of claim 1 wherein the dissection of the corneal endothelial cells comprises removal of the cells from the tissue source such that the cells are dissected away from the stroma of said tissue source prior to growing in the first culture system.

3. The method of claim 2 wherein said tissue source can be corneal buttons or rims.

4. The method of claim 2 wherein the ECM is comprised of Bovine corneal endothelial cell extracellular matrix (BCE-ECM).

5. The method of claim 2 wherein the ECM is comprised of an artificially generated extracellular matrix (AG-ECM).

6. The method of claim 4 wherein the Bovine corneal endothelial cell extracellular matrix (BCE-ECM) is made by the process comprising:

a) Bovine corneal endothelial cells (BCEC) are seeded onto dishes in DME-H16 medium containing approximately 10% Fetal Calf Serum, 5% Calf Serum, 5% Dextran, 300 mcg/ml glutamine, 2.5 mcg/ml Amphotericin B, and 50 ng/ml bFGF;

b) When the BCEC are confluent, the dishes will be treated with NH4OH at a volume sufficient to cover at least 2/3 of the plate for at least about 5 minutes whereafter the NH4OH removed;

c) The dishes will be stored at 4 °C about a week prior to use in order to

eliminate any surviving BCEC; and

d) Laminin, fibronectin, and type IV collagen will be added to the BCE-ECM plates prior to plating of the corneal endothelial cells.

7. The method of claim 5 wherein the artificially generated endothelial cell extracellular matrix (AG-ECM) is made by the process comprising:

a) fibronectin, laminin and RGDS (Arg-Gly-Asp-Ser peptide) are prepared in a 100 mcg/mL in distilled water;

b) collagen type IV is prepared at a concentration of about 1 mg/mL in 0.01% acetic acid;

c) basic fibroblast growth factor (bFGF) is prepared at a concentration of about 100 mcg/mL in bovine serum albumin (0.05% w/v);

d) the solutions of steps a, b, and c are mixed and then incubated at 4 °C for two hours; and

e) the mixture of step d is diluted about 1:10 with phosphate buffered saline, and then a sufficient amount of the solution is added to a dish and allowed to stand at 4 °C for approximately 1 hour before use.

8. Human corneal endothelial cells (HCEC) suitable for use in transplantation made using any one of the methods of claims 1 - 6.

9. An apparatus for growing cells in culture having at least one surface which is in contact with the cells and wherein the surface is coated with a mixture comprising BCE-ECM prior to use.

10. The apparatus of claim 9 selected from the group consisting of: cell culture plates and flasks.

11. The apparatus of claim 9 wherein the cells are mammalian cells.

12. An apparatus for growing cells in culture having at least one surface which is in contact with the cells and wherein the surface is coated with a mixture comprising AG-ECM prior to use.

13. The apparatus of claim 12 wherein the cells are mammalian cells.

14. A method of making HCEC cells wherein said HCEC are lacking class I HLA antigens comprising: dissecting human corneal endothelial cells from a neonatal source such that said cells do not express class I HLA antigens; growing said corneal endothelial cells at a low density in a range of about 100 to 500 cells per square millimeter in a primary culture system comprising extracellular matrix coated (ECM) culture plates for a period of time; passaging said cells into a secondary culture system wherein the secondary culture system is comprised of an ECM coated culture plates and the addition of sufficient cellular growth factors

until the cells are grown to confluency; and wherein said cells have grown to confluence and are then harvested from the second culture system in sufficient quantities to be useful in transplantation to a subject *in vivo*.

15. The method of claim 14 wherein the ECM is comprised of Bovine corneal endothelial cell extracellular matrix (BCE-ECM).

16. The method of claim 14 wherein the ECM is comprised of an artificially generated extracellular matrix (AG-ECM).

17. A method of making HCEC cells wherein said HCEC are lacking class I HLA antigens comprising: dissecting human corneal endothelial cells from a tissue source, growing said corneal endothelial cells at a low density in a range of about 100 to 500 cells per square millimeter in a primary culture system comprising extracellular matrix coated (ECM) culture plates for a period of time; passaging said cells into a secondary culture system wherein the secondary culture system is comprised of an ECM coated culture plates and the addition of sufficient cellular growth factors until the cells are grown to confluency; wherein said cells have grown to confluence and are then harvested from the second culture system in sufficient quantities to be useful in transplantation to a subject *in vivo*; transforming the cells such that the a cell line is created and said cells contain a targeted disruption in the HLA gene locus thereby inhibiting expression of HLA antigens.

18. The method of claim 1, 2 or 17, wherein the genotype of each HCEC cell line is determined using gel-based detection methods, using non-gel-based detection methods or with genetic markers.

19. The method of claim 1, 2 or 17, wherein the target immunotype of each HCEC cell line is determined using serological or molecular methods.

20. The method of claim 19, wherein the target immunotype is determined by HLA tissue typing.

21. A cell depository comprising multiple populations of HLA-typed HS HCEC cell lines, wherein each HCEC cell line is derived from a different donor and is homozygous for a unique HLA haplotype.

22. The cell depository of claim 21, wherein the HCEC cell lines are obtained from donors of different ethnicities.

23. The cell depository of claim 21 or 22, wherein the contents of the depository are catalogued.

24. A method for producing an HCEC cell depository of genotyped HCEC cells from multiple donors comprising: (a) selecting donors; (b) determining the genotype of each donor; (c) isolating HCEC cells from primary cultures obtained from each donor; (d) culturing the isolated HCEC cells to obtain HCEC cell lines; (e) determining the genotype of each HCEC cell line; and (f) cataloging the genotype of each HCEC cell line obtained in (g).

25. A method for producing a HCEC cell depository of immunotyped HCEC cells from multiple donors comprising: (a) selecting donors; (b) determining the immunotype of each donor; (c) developing primary cultures of HCEC cells; (d) isolating HCEC cells from each donor; (e) culturing the isolated HCEC cells to obtain HCEC cell lines; (f) determining the immunotype of each HCEC cell line; and (g) cataloging the immunotype of each HCEC cell line obtained in (e).

26. A method for producing a HCEC cell depository of genotyped and immunotyped HCEC cells from multiple donors comprising: (a) selecting donors; (b) determining the genotype and immunotype of each donor; (c) developing primary cultures of HCEC cells; (d) isolating HCEC cells from each donor; (e) culturing the HCEC cells to obtain HCEC cell lines; (f) determining the genotype and immunotype of each HCEC cell line; and (g) cataloging the genotype and immunotype of each HCEC cell line obtained in (e).

27. The method of claim 24, 25, or 26, wherein the donors are mammalian.

28. The method of claim 26, wherein the donors are human.

29. The method of claim 26, wherein the donors are non-human.

30. A method of transporting HCEC for transplantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC into a flask or suitable container filled with culture medium; and c) transporting said membrane coated with the HCEC.

31. The method of claim 31 wherein said target tissue is a corneal button.

32. The method of claim 31 wherein said target tissue is a secondary culture system.

33. A method of transporting HCEC for transplantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC onto a donor target tissue; c) growing the HCEC on the donor tissue for a time period sufficient to prevent dislodging during transportation; and d) transporting said tissue in a storage medium.

34. The method of claim 33 wherein the target tissue is a denuded cornea.

35. The method of claim 30 wherein the biodegradable polymer comprises a semi-solid state suitable for coating with BCE-ECM or other biocompatible coating such as Diamond-Like-Carbon.

36. A method for protecting the regenerated target tissue of claim 33 from denuding during transport or implantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC onto a donor target tissue; c) growing the HCEC on the donor tissue for a time period sufficient to prevent dislodging during transportation in the presence of Healon which has been

conjugated to bFGF; and d) transporting said tissue in a storage medium having Healon conjugated to bFGF.

37. The method of claim 36 wherein the target tissue is a denuded cornea.

38. The target tissue treated according to any one of claims 30, 33, 35, or 36.

39. A method of storing HCEC regenerated target tissue for transplantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC onto a donor target tissue; c) growing the HCEC on the donor tissue for a time period sufficient to prevent dislodging during transportation; d) addition of an anti-icing or cryoprotective agent to said tissue in a storage medium; and e) storage of the target tissue at a very low temperature.

40. A method for denuding a native cornea to make it suitable for transplantation or correction comprising the steps of:

a) a corneal button or other target tissue is placed in a suitable holder;

b) a sufficient quantity of denuding reagent is added to the holder in step a so that it completely covers the target tissue;

c) the tissue is incubated with the denuding reagent for a sufficient period of time at approximately room temperature; and

d) the target tissue is washed with an appropriate buffer approximately 10 times.

41. The method of claim 40 wherein the denuding reagent is comprised of a solution of Triton X at a concentration of about 0.01 to 1% v/v in phosphate buffered saline.

42. The method of claim 41 wherein the incubation time is about 5 minutes.

43. The method of claim 40 wherein the denuding reagent is comprised of a solution of ammonium hydroxide at a concentration of about 20 mM.

44. The method of claim 43 wherein the incubation time is between about 2 to 5 minutes.

45. A method for denuding a native cornea to make it suitable for transplantation or correction comprising the steps of:

a) a corneal button or other target tissue is placed in a suitable holder;

b) a sufficient quantity of distilled water is added to the holder in step a so that it completely covers the target tissue;

c) the tissue is incubated with the denuding reagent for about 15 minutes at

approximately room temperature;

d) about half the volume of water is aspirated off;

e) the wetted endothelium is the mechanically swept off from the corneal button; and

f) the corneal button is then washed approximately 3 times with phosphate buffered saline.

46. A reconstituted extracellular matrix preparation comprising: a sufficient amount of growth factor mixture and a sufficient amount of adhesion factor mixture.

47. The growth factor mixture of claim 46 comprising a sufficient quantity of bFGF, EGF and polycarbophyll in a suitable biological buffer.

48. The growth factor mixture of claim 47 wherein the concentrations of bFGF, EGF and polycarbophyll are approximately 3.33 mcg/mL, 33.33 mcg/mL and 0.33 mg/mL respectively.

49. The adhesion factor mixture of claim 46 comprising a sufficient quantity of laminin, fibronectin, RGDS, and collagen IV in a suitable biological buffer.

50. The growth factor mixture of claim 47 wherein the concentrations of laminin, fibronectin, RGDS, are approximately 83.33 mcg/mL, and collagen IV is approximately 250 mcg/mL.

51. A method of coating a denuded cornea comprising:

a) a corneal button or other target tissue is placed in a suitable holder;

b) the corneal button is then washed with phosphate buffered saline;

c) a sufficient quantity of reconstituted extracellular matrix preparation of claim 46 is added to the holder in step a so that it completely covers the target tissue;

d) the corneal button is incubated for a sufficient period of time at approximately 4 °C; and

e) the corneal button is then washed with phosphate buffered saline or other suitable buffer.

52. A method of coating a denuded cornea comprising:

a) a corneal button or other target tissue is placed in a suitable holder;

b) the corneal button is then washed with phosphate buffered saline;

c) a sufficient quantity of reconstituted extracellular matrix preparation of claim 46 is added to the holder in step a so that it completely covers the target tissue;

d) the corneal button is incubated for a sufficient period of time at approximately 4 °C;

e) the corneal button is then washed with phosphate buffered saline or other suitable buffer; and

f) approximately 300 mcL of Healon is added to the corneal button prior to seeding of new endothelial cells.

PTO/SB/01 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☑ Declaration Submitted With Initial Filing **OR** ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	
First Named Inventor	Ge Ming Lui
COMPLETE IF KNOWN	
Application Number	
Filing Date	October 10, 2003
Art Unit	
Examiner Name	

I hereby declare that:

Each inventor's residence, mailing address, and citizenship are as stated below next to their name.

I believe the inventor(s) named below to be the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Corneal Cell Culturing and Transplantation

(Title of the Invention)

the specification of which

☑ is attached hereto

OR

☐ was filed on (MM/DD/YYYY) ☐ as United States Application Number or PCT International Application Number ☐ and was amended on (MM/DD/YYYY) ☐ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? Yes	No
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☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR:	☐ A petition has been filed for this unsigned inventor		
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Inventor's Signature			Date
Residence: City	State	Country	Citizenship
Mailing Address			
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☐ Additional inventors or a legal representative are being named on the _____ supplemental sheet(s) PTO/SB/02A or 02LR attached hereto.

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